STUDIES ON THE CHEMISTRY OF ANAPHYLAXIS (II).*†

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IN a previous paper[1] were reported observations on the effects of tryptic digestion on the anaphylactic properties of bovine serum. It was found that as the proportion of coagulable nitrogen decreased, the power of the digestion mixture to intoxicate guinea-pigs sensitized with bovine serum, and to sensitize normal pigs to bovine serum, decreased in quite the same ratio. The results, as far as the experiments were recorded in that paper, were as follows: After 21 days' digestion of serum, when the coagulable nitrogen had been reduced to 8 per cent of the total nitrogen, the toxicity of the serum had been so nearly destroyed that 5 c.c. intraperitoneal doses caused no symptoms in sensitized pigs. It sensitized, however, in doses of 0.004 c.c. but not in doses of 0.0004 c.c., as contrasted with normal serum of which the minimal sensitizing dose is 0.0001 to 0.00001 c.c. The specificity of the reaction does not seem to be impaired by the digestion. A sample taken after 59 days' digestion contained 4.3 per cent of coagulable nitrogen, and its minimum sensitizing dose was 0.02 c.c. The toxicity was so reduced that 5 c.c. intraperitoneally, or 1 c.c. by intracardiac injection produced no symptoms in pigs sensitized with bovine serum; however, the same doses were toxic to pigs sensitized with the digestion mixture. Further digestion until the 129th day reduced the coagulable nitrogen to 2.5 per cent of the total nitrogen, and increased the minimum sensitizing dose to 0.1 c.c., without impairing the specificity.

Since that time the digestion has been continued, with the following results: After digestion from February 28 to November 1, nine months, the serum ("Digestion Mixture V") still gives a very faint turbidity on heating and acidifying, too small in amount to determine the nitrogen by the Kjeldahl method, and no distinct flocculi or other evidence of coagulable protein. The biuret reaction is still present but very faint. Injection into guinea-pigs gave the following results:

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[1] *Jour. Infect. Dis.*, 1908, 5, p. 449.

TABLE 1.

First Injection	Interval	Second Injection	Results
1. 0.1 c.c. bovine serum.....	23 days	5 c.c. digest. mixt. V*	No symptoms
2. 1.0 " digest. mixt. V.....	20 "	5 " " " "	" "
3. 0.1 " bovine serum.....	23 "	2 " " " "	(Intracardiac) slight symptoms
4. 0.1 " " "	23 "	1 " " " "	(Intracardiac) doubtful symptoms
5. 0.1 " " "	28 "	10 " " " "	No symptoms
6. 0.02 " digest. mixt. V.....	20 "	4 " bovine serum	" "
7. 0.05 " " " "	" "	4 " " "	" "
8. 0.1 " " " "	" "	4 " " "	" "
9. 0.5 " " " "	" "	4 " " "	No definite symptoms
10. 1.0 " " " "	" "	4 " " "	Severe symptoms (recovered)
11. 1.0 " " " "	" "	5	" " "

* Unless otherwise specified injections were made intraperitoneally.

Gave 3 c.c. bovine serum to each of Nos. 3, 4, and 5, 24 hours after the second injection, and all died with typical symptoms within one hour, showing absence of protective action by the digestion mixture.

The sensitizing power of the serum has, therefore, been so greatly reduced by nine months' digestion that the minimum sensitizing dose is 1 c.c., in contrast to the sensitizing dose of normal serum, which is one hundred-thousandth of a cubic centimeter, or less. The intoxicating power has been so far reduced that it cannot be detected by intracardiac injections of 1 to 2 c. c. or by intraperitoneal doses of 5 to 10 c.c. Larger doses cannot be used, as the digestion mixture possesses a certain degree of toxicity for normal pigs, which limits the size of the dose that may be used.

Digestion was continued until January 7 (314 days), and another sample taken. This sample gives a slight turbidity on neutralizing and heating, but no distinct biuret reaction. The results of injection into guinea-pigs were as follows:

TABLE 2.

First Injection	Interval	Second Injection	Results
1. 0.5 c.c. digest. mixt. VI....	17 days	3 c.c. bovine serum	Doubtful symptoms
2. 1.0 " " " "	17 "	" " " "	Died in 3 hours
3. 2.0 " " " "	19 "	" " " "	" " ½ "
4. 2.0 " " " "	19 "	" " " "	Slight symptoms
5. 2.0 " " " "	20 "	" " " "	" "
6. 4.0 " " " "	17 "	" " " "	Severe "
7 6.0 " " " "	17 "	" " " "	Moderate "
8. 10.0 " " " "	17 "	" " " "	Severe "
9. 2.0 " " " "	17 "	10 " digest. mixt. VI	Doubtful " *
10. 8.0 " " " "	17 "	" " " " "	" "
11. Normal pig.....	.....	" " " " "	Same as 9 and 10
12. " "	.....	" " " " "	" " " " "
13. 0.2 c.c. bovine serum.....	20 days	" " " " "	Doubtful symptoms
14. 0.1 " " "	22 "	" " " " "	" "
15. 2.0 " digest. mixt. VI.....	" "	" " " " "	" "
16. 2.0 " " " "	" "	" " " " "	" "
17. 0.1 " bovine serum.....	" "	2 " " " "	(Intracardiac) doubtful symptoms
18. 0.1 " " "	" "	" " " " "	" " "

* The pigs injected with the digestion mixture all became more or less sick, but the symptoms were not the same as in typical anaphylaxis, and normal pigs were fully as much affected as sensitized pigs.

Nos. 14, 17, and 18 were each given 4 c.c. bovine serum 24 hours after the second injection, and all three died with typical symptoms within 30 minutes.

Therefore the further digestion of this serum has not reduced the sensitizing power appreciably, the minimum dose still being about 1 c.c., altho the serum now has no distinct coagulability or biuret reaction. The digestion mixture is of itself quite toxic, but no more so for sensitized than for normal pigs, the symptoms resembling somewhat anaphylactic symptoms at first, but coming on without the usual period of incubation, and persisting 24 hours or more. As pigs that had been sensitized to bovine serum were killed by injection of bovine serum subsequent to injection of the digestion mixture, it is probable that symptoms produced by this digested serum are due to its own toxicity and not to any anaphylactic intoxication, for in the latter case the animals would have been refractory to bovine serum.

After removal of the sample used in the previous experiment (314 days' digestion), the remaining 200 c.c. of fluid was filtered free from precipitated substances, a fresh sample of trypsin (0.2 gm.) was added, and digestion was continued at 37° until July 6, 1909, a total period of over 16 months. It still gives a slight turbidity on neutralizing and heating, apparently due to traces of coagulable protein that cannot be removed by prolonged digestion, but no biuret reaction. When injected into guinea-pigs in doses of 1 to 5 c.c. it sensitizes them so that they react slightly, but typically, to bovine serum injected three weeks later, the most marked reaction being obtained in the pigs that have received the 5 c.c. doses; in no case was the reaction at all severe.

From these experiments with bovine serum it would seem that the anaphylaxis reaction depends upon some constituent of the serum that decreases in amount *pari passu* with the coagulable protein, but which persists after over a year's digestion with fresh quantities of trypsin. As the minimum dose of serum which will sensitize at this stage is about 1 or 2 c.c., and as the sensitizing dose of a pure protein (egg albumin) has been found to be as small as one-twentieth of a millionth of a gram, it is possible, and indeed probable, that the sensitizing power of the digested serum is due to the presence of coagulable protein which is still present in too small amounts to be detected by the heat test, which test is not adequate to detect any quantity as minute as this might be. The fact that the sensitizing and intoxicating power decrease together may be considered as

further evidence in support of the view maintained by Rosenau and Anderson, that the sensitizing and intoxicating principles of serum are one and the same, and not different proteins. However, it would be perfectly possible that two different proteins, one having sensitizing and one intoxicating properties, might be destroyed at approximately equal rates by digestion with trypsin.

An interesting phenomenon, which perhaps is of significance, is that when the serum has been digested to a point where it has but little toxicity for guinea-pigs sensitized with normal bovine serum, it is much more toxic for guinea-pigs that have been sensitized with the digested serum.[1] Apparently a special sensitization exists here, the guinea-pigs injected with digested serum being sensitized for undigested serum proteins by the still unaltered serum protein molecules, while at the same time they are sensitized specifically by some of the partially digested molecules for similarly altered molecules which are present in the digested serum and absent in the fresh serum. As subsequent experiments, to be described below, indicate that ordinary albumoses and peptones are not capable of either sensitizing or intoxicating guinea-pigs, in the sense of the anaphylactic reaction, it is probable that the molecules concerned in the above phenomenon are molecules of serum protein that have not been disintegrated quite as far as to the stage of albumoses. Obermayer and Pick observed a similar condition in their studies of the effects of tryptic digestion of serum upon its power to produce and to react with precipitins, for they found that the serum of rabbits immunized with such digested serum reacted only with the homologous digested serum, and not with normal serum.

DIGESTION OF EGG ALBUMIN BY TRYPSIN AND PEPSIN.

Experiments on the effects of tryptic digestion on crystallized egg albumin were not very successful, because the chloroform, which needs be added to prevent putrefaction, coagulates the albumin and thus destroys its sensitizing and intoxicating effects, which, as previously shown, are only produced when the protein is injected in a soluble form. No other antiseptic than chloroform seemed available, for it is the antiseptic that can be most readily removed from

[1] See Tables 13 and 14 of the previous paper.

the solution before injection, and, furthermore, egg albumin is coagulated by nearly all other antiseptics as much as or more than it is by chloroform. The following protocol will show the result of an experiment with tryptic digestion with egg albumin:

Dissolved 5 gm. crystallized egg albumin in 200 c.c. of 0.4 per cent sodium carbonate solution, and added 0.5 gm. active pancreatin and 5 c.c. chloroform. After standing four days at 37° the mixture showed a heavy precipitate, and the filtered fluid gave only a slight turbidity on being neutralized, boiled, and acidified with acetic acid. Injected this unfiltered mixture into pigs that had been sensitized 13 days previously with five milligrams of egg albumin each, with the following results:

1. Injected 5 c.c. of digestion mixture, but animal showed no distinct symptoms. Twenty-four hours later injected 0.15 gm. egg albumin, and the animal showed typical but not severe anaphylaxis symptoms.

2. Injected 10 c.c. of digestion mixture, and obtained slight but apparently typical symptoms. Forty-eight hours later gave 0.1 gm. egg albumin, which produced slight but typical symptoms.

3. Injected 10 c.c. of digestion mixture into a guinea-pig, which showed typical but not severe symptoms. Twenty hours later 0.15 gm. egg albumin was injected which killed the animal in 20 minutes.

For purpose of control a similar mixture was prepared with the pancreatin left out, and it was found to be of a jelly-like consistence (alkali albuminate) after standing five days in the incubator. Injected into pigs previously sensitized with albumin, the filtrate from this mixture produced much the same effects as recorded in the three previous experiments.

After the digestion mixture had been four days in the incubator it was left at room temperature for 13 days, and then a part was used for sensitization of four pigs, the rest kept in the ice box for later injections. The results of these injections were as follows:

1. Injected 2 c.c. digestion mixture, and 18 days later gave 0.1 gm. egg albumin. No distinct symptoms until after one hour, and then a slight degree of sickness and paralysis of the hind legs appeared, which persisted for 24 hours.

2. Injected 0.1 c.c. digestion mixture, and 18 days later gave 0.1 gm. egg albumin. Same effects as in No. 1, but less severe.

3. Injected 1 c.c. digestion mixture, and 19 days later gave 10 c.c. of the same mixture. Slight but typical symptoms after 15 minutes.

4. Injected 0.01 c.c. digestion mixture, and 19 days later gave 10 c.c. of same mixture. Slight but typical symptoms after 15 minutes.

From these experiments it is evident that the sodium carbonate and chloroform alter the egg albumin so much that any effect produced by the trypsin would be entirely masked. However, as shown by another series of experiments, digestion of 5 per cent egg albumin solution by trypsin for two months, when the filtrate gave only a faint biuret reaction, and no turbidity on neutralization and heating, rendered it so inert that even 10 c.c. doses did not sensitize guinea-

pigs in the least to egg albumin, and it had no intoxicating effect whatever upon pigs sensitized to egg albumin even when given by intracardiac injections. Apparently crystallized egg albumin is less resistant to tryptic digestion than is bovine serum, possibly because the former is deprived of antibodies in the process of crystallization, for antibodies are usually found to be contained in the globulin fraction of native proteins.

Peptic digestion of egg albumin was much more successful, for the antiseptic action of the hydrochloric acid was sufficient to prevent putrefaction without the addition of chloroform. The digestion mixture consisted of 5 gm. crystallized egg albumin and 0.5 gm. active pepsin (from pig) in 100 c.c. *n*/10 HCl. As Michaelis[1] found that the power of protein to react with precipitins is destroyed very quickly by peptic digestion (in 40 minutes in the case of serum), it was anticipated that a similarly rapid effect would be produced on the power of egg albumin to sensitize and to intoxicate, but such was not the case. At intervals 5 c.c. of the digestion mixture was pipetted off, and 5 c.c. *n*/10 NaOH added to neutralize it; the 10 c.c. of neutral mixture was then injected into pigs sensitized with egg albumin between two and three weeks previously, with the following results:

TABLE 3.

Duration of Digestion	Result of Injection into Sensitized Pigs	Remarks
1. 5 min.....	Death in 12 min.	.....
2. 30 "	" " 40 "	.....
3. 65 "	Severe symptoms	Recovered
4. 2 hours.....	Death in 40 min.	.....
5. 4 "	" " 85 "	.....
6. 7 "	" " 1½ hours	.....
7. 12 "	Severe symptoms	Recovered
8. 13 "	Died after 1½ hours	Died in night
9. 24 "	" in " "	*
10. 30 "	Slight symptoms	.....
11. 48 "	Doubtful "	†Symptoms apparently due to toxicity of digestion mixture rather than to anaphylaxis
12. 72 "	" "	.....

* Digestion mixture now (24 hours) yields but a very slight precipitate of acid albumin upon neutralization, but still considerable coagulable protein is present. This is, however, too small in amount to be determined by Kjeldahl's method in a 5 c.c. sample, there being not over 1 or 2 mg. of coagulable nitrogen.

† Solution after 48 hours' digestion showed only a mere trace of coagulable protein, but heavy biure reaction.

[1] *Ztschr. klin. Med.*, 1905, 56, p. 417.

This experiment indicates that peptic digestion of egg albumin destroys its power to intoxicate sensitized guinea-pigs only when it has destroyed practically all coagulable protein, indicating that this property of proteins is less readily modified than is the property of reacting with precipitins, according to the observations of Michaelis. This author also found that peptic digestion of serum until the coagulable protein was all removed, destroyed its power of acting as a precipitogen, showing that this property of protein, like its sensitizing property, is more resistant than its property of being precipitated by specific precipitins. In experiments recorded in the next table it is shown that the sensitizing power of egg albumin persists even after all positive evidence of coagulable protein has been lost, suggesting that the sensitizing agent is even more resistant than the precipitogens, possibly because larger amounts of precipitins must be present to be demonstrable. Pick and Yamanouchi,[1] failing to destroy the anaphylactic properties of serum by digestion 15 minutes with pepsin-HCl, conclude that this is evidence that the precipitin reaction and the anaphylaxis reaction are dependent on different substances, a conclusion which Michaelis[2] shows is unwarranted. As a matter of fact the experiments reported herewith show that the resistance of the sensitizing principle to pepsin is not very different from the resistance of the precipitogens as determined by Michaelis, if we take into consideration that a considerable amount of precipitogen must be present in a solution to be demonstrable by its production of precipitins, while sensitization may be accomplished by the merest traces of protein.

There is a little question concerning the influence of the pepsin in these experiments, for it was found that the conversion of albumin into acid-albumin had a slight effect upon its power to intoxicate sensitized guinea-pigs, as shown by the following experiment:

One gram crystallized egg albumin was dissolved in 20 c.c. water, and 3 c.c. of 20 per cent HCl added. This caused a heavy precipitate, and the mixture was left standing at room temperature over night. It was then filtered, washed, and the precipitate was suspended in 20 c.c. of water and dissolved in a minimum amount of NaOH. This solution was then injected in doses of from 3 to 5 c.c. into four guinea-pigs that had been sensitized two weeks previously, causing the death of one, severe symptoms in another,

[1] *Ztschr. f. Immunitätsforschung*, 1909, 1, p. 676.

[2] *Ibid.*, 1909, 2, p. 29.

but only slight symptoms in the other two. The two last pigs were injected 48 hours later with egg albumin, one reacting severely, the other but slightly.

Four animals were also sensitized with the acid albumin solution, in the following doses: No. 1, 5 c.c.; No. 2, 2 c.c.; No. 3, 1 c.c.; No. 4, 0.1 c.c. Each was injected with 0.15 gm. albumin 19 days later with the following results: No. 1 showed slight symptoms; No. 2 became severely sick but recovered; Nos. 3 and 4 died each in 35 minutes.

Apparently, then, acid albumin is somewhat less effective in intoxicating pigs sensitized to natural albumin than is the natural albumin itself, but it by no means loses either its intoxicating or sensitizing effects. Converting egg albumin into alkali albuminate, however, seems to have a much more marked influence upon its anaphylactic activity. Egg albumin converted into alkali albuminate, precipitated with acetic acid, washed, and redissolved in alkali, caused in 0.1 gm. doses absolutely no symptoms in guinea-pigs that had been sensitized to egg albumin, and left them just as sensitive to egg albumin as control pigs.

Even prolonged digestion with pepsin, beyond the time of disappearance of recognizable coagulable protein and acid albumin, does not entirely destroy the sensitizing power of egg albumin, as shown by the following table:

TABLE 4.

Duration of Digestion	Result of Injection of Egg Albumin	Condition of Digestion Mixture Used for Sensitizing
1. 24 hours	Death in 20 min.	Some coagulable protein
2. 48 "	" " " "	Trace of " "
3. 72 "	" " 15 "	" " " "
4. 4 days	" " 15 "	Faint turbidity on heating
5. 5 "	Slight symptoms	" " " "
6. 6 "	Moderate "	" " " "
7. 8 "	" "	Trace of " " "
8. 12 "	Slight "	Doubtful " " "
9. 18 "	No "	" " " "
10. 26 "	Moderate "	No " " "
11. 36 "	Died in 40 min.*	" " " "
12. 36 "	Doubtful symptoms	" " " "

* This exceptional result may be due to a sensitized pig having been accidentally used.

The results of the foregoing experiments with digestion mixtures, indicating that the anaphylaxis reaction depends either upon whole protein molecules, or at least on molecules but slightly altered from the native coagulable state, did not make it probable that the isolated cleavage products of the proteins would be found to be actively anaphylactic, nevertheless a number of preparations of albumoses

and peptones were made from egg albumin and tested on guinea-pigs, as shown by the following summaries:[1]

A. Albumose was prepared from raw egg white by digestion with pepsin-HCl, removal of coagulable proteins, precipitation by saturation with ammonium sulfate dialysis, and carefully purified by twice reprecipitating. In doses of 0.25 gm. and over this caused the animals to become slightly sick, but doses as high as 0.5 gm. did not usually produce serious effects. Several guinea-pigs were injected with from 0.05 to 0.5 gm. of this albumose, and after 14 days given 0.1 gm. doses of egg albumin, or 0.3 gm. doses of albumose. A few of the animals showed slight atypical symptoms, but no conclusive evidence of anaphylaxis was obtained. Guinea-pigs sensitized with egg albumin were found not susceptible to the albumose, and injection of albumose left them still susceptible to egg albumin.

B. Albumose prepared from coagulated egg white in the same way as above, was equally inert, neither sensitizing pigs to egg albumin or albumose, nor intoxicating pigs that had been previously given sensitizing doses of albumin or albumose. As with albumose **A**, pigs sensitized to egg albumin, and injected after two weeks with the albumose preparations, were still quite as susceptible to a second dose of egg albumin as if they had not received the albumose injection, indicating that the albumose from egg albumin causes no reaction whatever in animals sensitized to egg albumin.

C. A series of fractions of the products of digestion of raw egg white by an extract of fresh pig pancreas was prepared, including the ordinary peptones, and several fractions of the crystalline and non-crystalline products. None of these had any power of sensitizing guinea-pigs to itself or to egg albumin, or of intoxic ating guinea-pigs that had been sesitized to egg albumin, or of rendering such sensitized animals refractory to egg albumin.

The results of the foregoing experiments leave little room for doubt that the anaphylaxis reaction can be produced only by intact protein molecules, or at least by molecules of proteins less removed from the whole proteins than are the ordinary albumoses and peptones. That some of the first products of protein cleavage may have sensitizing powers for the whole protein molecules is suggested by the following experiments:

A. Dissolved 3 gm. crystallized egg albumin in 150 c.c. water, heated it to 100° for 45 minutes, added a drop of dilute acetic acid, filtered, and washed. Ground the coagulum in a mortar, added 60 c.c. of 0.5 per cent sodium carbonate solution, 0.4 gm. pancreatin, and 2 c.c. chloroform. Kept at 38° C. After varying periods took homogenous samples, filtered, and injected the filtrate into guinea-pigs, with the following results:

1. Forty-eight hours' digestion. Filtrate from a 10 c.c. sample injected into guinea-pig previously sensitized with egg albumin. Animal became slightly sick, but symptoms were not altogether typical of anaphylaxis; apparently they were due rather to the toxicity of the mixture, as 48 hours later an injection of 0.15 gm. egg albumin caused death in 35 minutes.

[1] Most of these preparations were made in the Sheffield Biological Laboratory of Yale University to the staff of which I am indebted for much advice and assistance.

A second 5 c.c. sample was injected into a normal guinea-pig, and 15 days later this animal was given 0.1 gm. egg albumin which caused severe, typical, but not fatal symptoms.

2. Five days' digestion. Ten c.c. sample caused no distinct symptoms in pig sensitized to egg albumin; and the animal reacted typically but not fatally to egg albumin injected 4 hours later.

A normal guinea-pig given 5 c.c. of this digestion mixture reacted slightly but typically when injected with egg albumin 14 days later.

3. Seven days' digestion. No distinct symptoms caused by 10 c.c. injected into a sensitized guinea-pig, and injection of egg albumin 4 hours later caused the death of the animal in 30 minutes.

4. Twelve days' digestion. Five c.c. sensitized a guinea-pig so that when injected with egg albumin 17 days later it became severely and typically sick.

5. Twenty-one days' digestion. Two guinea-pigs receiving 5 c.c. of this material showed no symptoms on subsequent injection with egg albumin.

B. Dissolved 2 gm. crystallized egg albumin in 150 c.c. water, and heated at 100° for one hour. Filtered, and injected the clear, water-like filtrate, after concentration to 10 c.c., into a normal guinea-pig. Twenty-two days later the guinea-pig reacted typically, altho not severely, to an injection of egg albumin.

Ground up the coagulum, added 40 c.c. *n*/10 HCl and 0.1 gm. pepsin, and kept at 37°. At intervals of 24, 48, 100, and 150 hours withdrew 5 c.c. samples, neutralized with 5 c.c. *n*/10 NaOH, and injected the mixture without filtration into normal guinea-pigs. All four pigs reacted typically when injected with egg albumin 15 days later, none fatally, but the pig receiving the 24-hour sample became extremely sick. When injected into pigs sensitized with egg albumin this digestion mixture produced no definite symptoms.

From these experiments it would seem that some of the products of hydrolysis of coagulated egg albumin have to a slight extent the power of sensitizing to egg albumin. Either the amount of these sensitizing substances is exceedingly minute, or else they do not sensitize very effectively to the unaltered egg albumin, for the sensitized guinea-pigs never reacted fatally and only occasionally was the reaction severe. Corresponding to this feeble sensitizing power, the intoxicating effect of the products of digestion of boiled egg albumin upon sensitized pigs is practically nothing.

SPECIFICITY OF THE PROTEINS OF EGGS.

Among the various proteins that have been isolated from eggs, besides the albumin, are ovomucoid and ovovitellin. The results of experiments with ovomucoid are shown in Table 5, p. 516.

From these experiments a number of interesting facts may be learned. In the first place the fact that ovomucoid intoxicates severely, sometimes fatally, guinea-pigs sensitized with ovomucoid,

is most conclusive proof of the opinion supported in the previous article, that the effects of heat upon the substances concerned in the anaphylaxis reaction with serum and egg albumin are due solely to the coagulation of the proteins. Ovomucoid is prepared by heating solutions of egg white to boiling to coagulate all coagulable proteins; the filtrate, usually many liters in amount, is evaporated over the flame or on the water bath to a small volume, and the ovomucoid precipitated with alcohol, redissolved in water, and reprecipitated. During this process the ovomucoid is subjected to a temperature at or near the boiling point for some hours, and also is precipitated with

TABLE 5.

First Injection	Interval	Second Injection	Results
1. 0.0025 gm. ovomucoid.....	19 days	0.25 gm. ovomucoid	Died in 7 hours
2. 0.0005 " "	" "	0.25 " "	Severe typical symptoms
3. 0.0005 " "	" "	0.25 " "	Slight " "
4. 0.0005 " "	" "	0.20 " "	Severe " "
5. 0.0005 " "	" "	0.1 " egg albumin	No distinct symptoms
6. 0.012 " "	22 "	0.15 " " "	Slight symptoms*
7. 0.012 " "	" "	0.1 " ovomucoid	Died in 35 minutes
8. 0.005 " "	16 "	0.1 " "	Slight symptoms
9. 0.0005 " "	" "	0.1 " "	" "
10. 0.00005 " "	" "	0.1 " "	" "
11. 0.005 " "	" "	0.15 " egg albumin	Very slight symptoms
12. 0.0005 " "	" "	0.15 " " "	" " "
13. 0.00005 " "	" "	0.15 " " "	" " "
14. 0.005 " egg albumin.....	30 "	0.2 " ovomucoid	No symptoms
15. 0.005 " "	" "	0.2 " "	" " †
16. 0.005 " ovovitellin.....	20 "	0.25 " "	" " †

* Twenty-four hours later received 0.1 gm. ovomucoid and became moderately but typically sick.

† Twenty-four hours later injection of 0.1 gm. egg albumin into Nos. 14 and 15 caused death in less than 15 minutes.

alcohol several times, yet in spite of this treatment it is able to cause typical anaphylaxis effects. Unquestionably the persistence of anaphylactic activity in ovomucoid in spite of all this heating and precipitating, depends upon the fact that ovomucoid does not lose its solubility in water because of these manipulations.

Also, the experiments on cross-sensitization of crystallized egg albumin and ovomucoid show that each of these substances is specific for itself, altho both come from a common source, the hen's egg. This is an important point in view of the unsettled state of our information concerning species specificity, as indicated by the precipitin test. The commonly accepted statement is that precipitins are specific for the species that furnishes the antigen, but that they will not distinguish between two different proteins from the same species.

There is, however, some evidence that precipitins can be obtained that will distinguish at least quantitatively between two different proteins from the same species, but the evidence is not altogether harmonious. The results here recorded with the ovomucoid are so important that the work will be extended later when a larger amount of ovomucoid has been collected.[1] It may be mentioned in this connection that Rosenau and Anderson[2] were able to sensitize guinea-pigs with guinea-pig placenta, a fact which, in conjunction with the ovomucoid results, suggests that the specificity exhibited by the anaphylaxis reaction may be somewhat different from the specificity shown by the precipitin reaction.

Similar indications of specificity were obtained in a few experiments with ovovitellin from the hen's egg, as follows:

TABLE 6.

First Injection	Interval	Second Injection	Results
1. 0.005 gm. ovovitellin.....	20 days	0.25 gm. ovomucoid	No symptoms
2. 0.005 " "	" "	0.1 " egg albumin	Slight "
3. 0.005 " "	" "	ovovitellin*	Death, one hour
4. 0.005 " "	" "	"	Moderate symptoms
5. 0.05 " "	" "	0.15 gm. egg albumin	Slight "
6. 0.05 " "	" "	ovovitellin	Died in 45 minutes

* This preparation of ovovitellin, for which I am indebted to Dr. T. B. Osborne of New Haven, is not very soluble in weak alkalies on account of frequent extraction with alcohol for the purpose of removing lecithin. Consequently, the exact dosage was not readily determined, and in these experiments where the dosage is not specified 10 c.c. of 0.2 per cent NaOH solution saturated with ovovitellin was used, probably containing from 0.05 to 0.1 gm. of protein.

RELATION OF CLEAVAGE PRODUCTS OF PROTEINS TO THE ANAPHYLAXIS REACTION.

Recently Biedl and Kraus[3] have reported studies on anaphylaxis in dogs, which led them to conclusions which seem surprising to one accustomed to working with guinea-pigs. Dogs do not react violently to a second dose of serum, and have not generally been considered suitable for anaphylaxis experiments, but Biedl and Kraus find that there is a marked fall of blood pressure when foreign serum is injected into sensitized dogs, which is not observed when the injection is made into normal dogs. This fall of blood pressure seems to depend upon a primary peripheral vaso-dilatation, and these authors con-

[1] For the preparation used in the above experiments I am indebted to Mr. M. S. Fine of the Sheffield Biological Laboratory.

[2] *Bulletin 45*, Hygienic Laboratory, June, 1908.

[3] *Wien. klin. Wchnschr.*, 1909, 22, p. 363.

sider that it is the chief phenomenon in the anaphylaxis reaction. Accompanying the fall of blood pressure is a decrease in the coagulability of the blood, and as these two phenomena are also produced by "peptone" they sought for a relationship between anaphylaxis and peptone intoxication. This they believe they have established by the following observations:

The symptoms following the injection of Witte's peptone into dogs in intravenous doses of 0.3 to 0.03 gm. per kilo are the same in all respects as the symptoms of anaphylaxis in dogs; if Witte's peptone is given to dogs sensitized to serum (horse or bovine) they react in the same way to it as do normal dogs, but are thereby made insusceptible to a subsequent injection of serum; in other words, a sensitized dog reacting to peptone becomes refractory to serum, exactly as if the second dose had been serum rather than peptone. On the other hand, animals given two doses of serum, and reacting after the second, may then either react or fail to react to a subsequent dose of Witte's peptone. They quote the old observations of Pick and Spiro that proteoses (Witte's "peptone" consists chiefly of proteoses) are not themselves toxic and that the observed toxicity is due to contaminating substances, being unfamiliar with the later work of Underhill[1] which seems to establish that proteoses are themselves toxic. They therefore conclude that the anaphylactic reaction is induced by some poison which is related to or identical with the poisonous element of Witte's peptone, a conclusion quite similar to that propounded some time ago by Vaughan, with whose work Biedl and Kraus seem to be unacquainted.

Richet[2] has criticized the article of Biedl and Kraus at some length, having himself observed in his actinotoxin experiments the fall in blood pressure, but not the loss of coagulability. He agrees with them that the action is not upon the heart, but doubts the reliability of their conclusion that the vaso-dilatation is not a secondary phenomenon due to disturbance of the central nervous system. As he points out, the fall of blood pressure of itself is not sufficient to account for all the features of anaphylaxis, for in the first place an equally great fall of blood pressure produced by amyl nitrite or other

[1] *Amer. Jour. Physiol.*, 1903, 9, p. 345

[2] *Presse Méd.*, 1909, 17, p. 249.

means does not cause any such profound effects; and secondly, there are many irritative and paralytic phenomena which are obviously entirely independent of the lowered blood pressure. Furthermore, the anaphylactic reaction is specific, while the peptone reaction is not, and in addition, the quantity of "peptone" necessary to produce symptoms is far greater than the quantity of protein necessary to call forth violent manifestations of anaphylaxis.

It should also be suggested that the results obtained by observing changes in the blood pressure in dogs are by no means comparable with results obtained with guinea-pigs, upon which most of the work so far reported has been done, since in these animals the symptoms are entirely different from the symptoms in the dog, and much more closely resemble the effects seen in man.[1] Indeed, even in the guinea-pig the symptom complex varies distinctly and strikingly with the protein used, so that it is possible to tell with some assurance whether a reaction is being produced with egg white, which causes highly irritative effects, or with serum, which causes more paralytic symptoms. One may also comment on the drawing of conclusions from results obtained with a substance of so uncertain and unknown composition as Witte's peptone.

In any event, the crucial point of Biedl and Kraus's proof of the relationship of anaphylaxis to peptone intoxication, namely, that injection of peptone into dogs sensitized to serum renders them refractory to serum, is not in harmony with results obtained in experiments which have been carried out with guinea-pigs, in conjunction with the studies of the relation of cleavage products of proteins to anaphylaxis reported in this and the previous paper. Numerous observations have been made showing that guinea-pigs sensitized to serum and egg albumin are not rendered refractory to the homologous protein by injection of either the entire products of digestion of these proteins by pepsin or trypsin, or by the isolated fractions of the digestion products. Furthermore, the various

[1] As having an important bearing on the results of Biedl and Kraus, may be quoted from Underhill's article the following statements as to the influence of species upon the effects of proteoses: "The organism of the dog is particularly susceptible to the effects of intravenous injections of the products of proteolysis. In the cat the characteristic symptoms are evoked somewhat less readily, larger doses being necessary to produce comparable results. The rabbit, on the other hand, is extremely resistant, and, except in rare cases, fails to respond in so far as the phenomena involving the blood are concerned. . . . . Although commercial 'propeptone' preparations are fatal to guinea-pigs they fail to render the blood incoagulable except when injected rapidly into fasting animals."

cleavage products and digestion mixtures do not produce in either normal or sensitized guinea-pigs the symptoms characteristic of anaphylaxis. Even when given in lethal doses the symptoms are entirely dissimilar, and death occurs after many hours, even days, unlike the sharp violent anaphylaxis reaction.

The lack of influence of products of protein digestion upon anaphylaxis is shown in the following table:

TABLE 7.

	Sensitizing Substance	First Intoxicating Dose	Result	Second Intoxicating Dose	Result
1.	Egg albumin...	Albumose "A"	No symptoms	Egg albumin	Death
2.	" " ...	" "A"	" "	" "	"
3.	" " ...	" "B"	" "	" "	"
4.	" " ...	" "B"	" "	" "	"
5.	" " ...	Peptone (tryptic)	" "	" "	"
6.	" " ...	" "	" "	" "	"
7.	" " ...	" (peptic)	" "	" "	Slight symptoms
8.	" " ...	" "	" "	" "	Severe "
9.	" " ...	" "	" "	" "	Death
10.	" " ...	" "	" "	" "	"
11.	" " ...	Crystalline products of tryptic digestion	" "	" "	"
12.	" " ...	Same as No. 11	" "	" "	"
13.	" " ...	Last fraction of tryptic digestion	" "	" "	"
14.	" " ...	Same as No. 13	" "	" "	"
15.	" " ...	Entire products of tryptic digestion of coagulated egg albumin	" "	" "	"
16.	" " ...	Same as No 15	" "	" "	Severe symptoms
17.	" " ...	Same as No. 15	" "	" "	Death
18.	" " ...	Digestion of raw egg white (tryptic)	" "	" "	Moderate symptoms
19.	" " ...	Same as No. 18	Doubtful "	" "	" "
20.	" " ...	Same as No. 18	No "	" "	Death
21.	" " ...	Same as No. 18	" "	" "	"
22.	Bovine serum ..	Digestion mixture V	" "	Bovine serum	Moderate symptoms
23.	" " ..	" " "	" "	" "	Death
24.	" " ..	" " "	" "	" "	"
25.	" " ..	" " VI	" "	" "	"
26.	" " ..	" " "	" "	" "	"
27.	" " ..	" " "	" "	" "	"
28.	" " ..	" " VIA	Doubtful "	" "	Moderate symptoms
29.	" " ..	" " "	" "	" "	" "

NOTE.—The digestion products mentioned in the above table may be described briefly as follows: Albumose "A" is the mixture of albumoses obtained from coagulated egg white by peptic digestion, removal of coagulable protein, precipitation by saturation with ammonium sulfate; dialysed, and purified by being twice precipitated with ammonium sulfate and dialyzed. Albumose "B" is a similar product from peptic digestion of raw egg white. Peptone "tryptic" and "peptic" are the alcohol precipitable fractions from tryptic and peptic digestion of raw egg white, after removal of albumose, purified by dialysis. After removal of the peptone from the tryptic digestion mixture, several crops of crystalline material were obtained (Experiments 11 and 12), and after these were removed a non-crystalline mass was left (Experiments 13 and 14). In Experiments 15 to 21 egg albumin was digested with trypsin until free from coagulable protein, and the entire mixture injected. Digestion mixtures V, VI, VIA are the entire products of tryptic digestion of bovine serum for various periods of time, as described previously in this article.

SUMMARY.

It is extremely difficult to digest serum entirely free from coagulable protein by trypsin, a specimen digested over 16 months still showing possible traces of coagulable material. Such serum in doses of 1 c.c. and over still sensitizes guinea-pigs to the homologous protein, altho not fatally. The intoxicating power disappears before the sensitizing power, corresponding to the much smaller dose of the latter which is necessary to produce effects. Digestion of serum does not affect its specificity, but partially digested serum is more toxic to guinea-pigs sensitized with the digested serum than to pigs sensitized with normal serum. Trypsin destroys the sensitizing and intoxicating power of crystallized egg albumin much more readily than it affects serum, presumably because this purified albumin is free from globulins, which are the proteins of serum and tissues that are most resistant to trypsin. Pepsin-HCl digestion of egg albumin destroys its sensitizing and intoxicating properties very slowly, the former persisting to a slight degree even after coagulable protein cannot be longer demonstrated. Conversion of egg albumin into acid albumin somewhat impairs, but by no means destroys, its power to sensitize guinea-pigs to egg albumin and to intoxicate pigs that have been sensitized with egg albumin. Conversion of egg albumin into alkali albumin, however, renders it entirely inert to pigs sensitized with egg albumin.

The products of digestion of egg white by pepsin or trypsin which can be separated by the ordinary methods, i. e., albumoses, peptones, polypeptids, crystallizable amino-acids, etc., have no power to sensitize or intoxicate guinea-pigs, whether used in conjunction with themselves or with undigested egg white.

These experiments indicate that proteins cannot be decomposed much if any beyond the coagulable form without losing their anaphylactic properties. For the anaphylaxis reaction we must have intact protein molecules in soluble form. Possibly there exists a stage immediately between the entire protein molecule and the ordinary proto- and deutero-proteoses, with which anaphylaxis can be produced, for coagulated proteins digested with either pepsin or trypsin show at certain stages a slight power to sensitize animals to egg albumin, but the power is very slight.

Preliminary experiments indicate that ovomucoid (and possibly also ovovitellin) has a specific anaphylactic action, reacting only with itself and not with other proteins of egg white. This form of specificity, i. e., a protein causing reaction with its own kind and not with other kinds of protein from the same species of animal, has not been conclusively demonstrated with the precipitin reaction, and is of so much importance in the larger problems of biological specificity that it will be investigated further. As ovomucoid causes fatal anaphylaxis reactions after having been heated for several hours at or near the boiling point, and after being precipitated and reprecipitated with alcohol, we have conclusive evidence that moist heat affects the anaphylactic property of proteins only when it renders the proteins insoluble.

The evidence adduced by Biedl and Kraus that in dogs the anaphylactic reaction is produced by substances related to Witte's peptone finds no confirmation in experiments performed with guinea-pigs. They observed that Witte's "peptone" administered to dogs sensitized to horse serum rendered the dogs refractory to subsequent injections of horse serum, as shown by blood pressure measurements; but in guinea-pigs entire digestion mixtures, or isolated fractions of digestion products, did not render sensitized pigs refractory to the homologous proteins. Apparently there are marked differences in the anaphylaxis reaction in different animals, and there seem to be some differences in the reactions produced by different proteins.